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Development of gene transfer systems in *Methylobacillus flagellatum* KT: Isolation of auxotrophic mutants

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Abstract. A collection of polyauxotrophic mutants of the obligate methylotroph *Methylobacillus flagellatum* KT was obtained. On the first step two stable auxotrophic mutants with a high requirement for amino acids supplements were isolated by treatment with nitrosoguanidine and selection on complete medium. Spontaneous variants of these mutants with a low requirement for nutrient supplements were the base for obtaining polyauxotrophic strains. It was shown, that the growth of mutants of *M. flagellatum* KT is inhibited by complete medium. Some amino acids and nucleotides are the inhibitor components of complete media. An approach for selection of auxotrophic mutants of individual genes was worked out on minimal medium. The optimal conditions for nitrosoguanidine mutagenesis of *M. flagellatum* KT were developed. The possible mechanisms of action of some of the nutrient supplements on the growth of *M. flagellatum* KT are discussed.

Key words: *Methylobacillus flagellatum* KT — Obligate methylotroph — Nitrosoguanidine mutagenesis — Auxotrophic mutants

Despite the considerable number of genetic studies which have been carried out on methylotrophic bacteria, progress in the genetic analysis of methylotrophs has been slow (de Vries 1986). The first step in genetic investigation of any microorganism involves the isolation of auxotrophic mutants. The isolation of mutants in methylotrophs and especially in obligate methylotrophic bacteria has not proved as easy as with heterotrophic bacteria. The isolated mutants of facultative methylotrophs included auxotrophs with leaky phenotype and a number of mutants unable to grow on C-1 compounds (O'Connor et al. 1977; O'Connor and Hanson 1978). For *Methylophilus methylotrophus*, an obligate methylotroph, using N-methyl-N'-nitro-N-nitrosoguanidine temperature-sensitive mutants have been obtained, including auxotrophs (Windass et al. 1980). Nevertheless, using a variety of methylotrophs and a broad range of mutagens, including UV irradiation, nitrosoguanidine, methyl methane sulphonate and ethyl methane sulphonate the mutagenesis procedures have not been developed for effective isolation of mutants in methylotrophs.

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A new approach for isolation of auxotrophic mutants in the facultative methylotroph *Methylobacterium* AM1 was developed by Whitta et al. (1985). Auxotrophic mutants and mutants involving C-1 metabolism have been obtained using transposon Tn5 mutagenesis.

The reasons why some of the chemical mutagens are not effective in the isolation of mutants in methylotrophs are still not entirely clear. It has been suggested that the absence of permeability for metabolites and therefore the death of potential auxotrophic mutants could be responsible for the lack of mutagenesis in methylotrophs (Haber et al. 1983). A lack of an SOS DNA repair system may also be the reason for lack of response to UV irradiation and other mutagens (Higgins et al. 1981; Williams and Shimmin 1978). The limited range of auxotrophs found may result also from the selection procedures used including plating of mutagenised cells on complete media and inhibition of auxotrophs by individual amino acids (Whitta et al. 1985).

In order to overcome the problems involved in carrying out genetics in obligate methylotrophs we have developed nitrosoguanidine induced mutagenesis for *Methylobacillus flagellatum* KT (Govorukhina et al. 1987). This strain is an obligate methylotroph which assimilates methanol by KDPGA/TA variant of ribulose monophosphate pathway. Preliminary results on isolation of mutants were reported at the C-1 Symposium in Holland, 1986.

Materials and methods

Bacterial strains

The strains of *Methylobacillus flagellatum* KT obtained and employed in the present study are given in Table 1. *Media and conditions of cultivation.* For growing strains of *M. flagellatum* KT we used minimal salts M9 medium (Maniatis et al. 1982) and complete LB medium (Miller 1972) with 2% methanol as a source of carbon and energy (M9M and LBM, respectively). Solid media contained 1.5% agar. For growth stimulation sodium-pyruvate was added to solid media M9M to a final concentration of 0.1% (M9MP). Nutrients required for growth of auxotrophic mutants were added at concentrations of: 400–500 μg/ml for strains with high requirement of amino acids; 200 μg/ml threonine, tryptophan, adenine for corresponding auxotrophic mutants and 200 μg/ml histidine for mutants MFK16 and MFK17; 200 μg/ml tyrosine for mutant MFK50; 10 μg/ml of biotin for biotin-requiring strains and 100 μg/ml of the remaining amino acids. Strains resistant to streptomycin (Sm), nalidixic acid (Nal) and rifampicin (Rif) were grown

Table 1. Strains of *Methylobacillus flagellatum* KT

Strain[a]	Genotype[b]	Derivation[c], origin
MFK1	prototroph, wild type	Govorukhina et al. 1987
MFK2	prototroph *str*-1	MFK1, spontaneous for *str*-1
MFK3H	*leu*-1 *str*-1	MFK2
MFK3	*leu*-1 *str*-1	MFK3H, spontaneous for lowered requirement of leucine
MFK4	*leu*-1 *str*-1 *rif*-1	MFK3, spontaneous for *rif*-1
MFK5	prototroph *str*-1	MFK3, spontaneous revertant
MFK6H	*vil*-1	MFK1
MFK6	*vil*-1	MFK6H, spontaneous for lowered requirement of amino acids
MFK7H	*trp*-1 *str*-1	MFK2
MFK8H	*met*-5 *str*-1	MFK2
MFK9H	*met*-6 *str*-1	MFK2
MFK10	*vil*-1 *met*-1 *nal*-1	MFK6, spontaneous for *nal*-1, NTG for *met*-1
MFK11	*leu*-1 *thr*-1 *str*-1	MFK3
MFK12	*leu*-1 *thr*-2 *str*-1	MFK3
MFK13	*leu*-1 *hisB*1 *str*-1	MFK3
MFK14	*leu*-1 *hisB*1 *str*-1 *rif*-5	MFK13, spontaneous for *rif*-5
MFK15	*leu*-1 *his*-3 *str*-1	MFK3
MFK16	*leu*-1 *his*-4 *str*-1	MFK3
MFK17	*leu*-1 *his*-6 *str*-1	MFK3
MFK18	*leu*-1 *pheA*1 *str*-1	MFK3
MFK19	*leu*-1 *ilvA*1 *str*-1	MFK3
MFK20	*leu*-1 *met*-2 *str*-1 *rif*-1	MFK4
MFK21	*leu*-1 *met*-3 *str*-1 *rif*-1	MFK4
MFK22	*leu*-1 *trpE*1 *str*-1	MFK3
MFK23	*leu*-1 *trp*-3 *str*-1	MFK3
MFK24–MFK40 (16 strains)	*leu*-1 *bio*-1 *str*-1/*leu*-1 *bio*-16 *str*-1	MFK3
MFK41	*pur*-2 *str*-1	MFK3
MFK42	*pur*-3 *str*-1	MFK3
MFK43	*leu*-1 *hisB*1 *met*-4 *str*-1 *rif*-5	MFK14
MFK44	*leu*-1 *hisB*1 *met*-4 *ala*-1 *str*-1 *rif*-5	MFK43
MFK45	*leu*-1 *hisB*1 *met*-4 *ilvA*2 *str*-1 *rif*-5	MFK43
MFK46	*leu*-1 *hisB*1 *met*-1 *ilv*-1 *str*-1 *rif*-5	MFK43
MFK47	*leu*-1 *pheA*2 *str*-1 *rif*-5	MFK43
MFK48	*leu*-1 *pheA*3 *str*-1 *rif*-5	MFK43
MFK49	*leu*-1 *hisB*1 *met*-4 *tyr*-3 *str*-1 *rif*-5	MFK43
MFK50	*leu*-1 *hisB*1 *met*-4 *pheA*4 *tyr*-4 *str*-1 *rif*-5	MFK43

[a] Auxotrophic mutants with high requirement of amino acids are designated with index "H"-high

[b] Genotype symbols are the same as those used for *Escherichia coli* K-12 (Bachmann 1983); exception are; *str*, streptomycin resistance; *vil*, requirement for valine, isoleucine and leucine (Holloway and Morgan 1986)

[c] All auxotrophic mutants were obtained after treatment of the parent strain indicated with NTG

on media supplemented with 300 μg/ml Sm, 20 μg/ml Nal and 100 μg/ml Rif, respectively. Cells were cultured at 42° C, and vigourouly aerated, when in liquid media. These conditions and growth on liquid medium M9M provide a generation time of 1–2 hours for MFK1, and for strains with one or two auxotrophic mutations, compared to 3–4 h for strains with three and four auxotrophic mutations.

Treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

For optimizing the conditions of NTG-mutagenesis the auxotrophic mutants of *M. flagellatum* KT were grown for 16–18 h on M9M medium, with the corresponding supplements, up to a density of $6-8 \times 10^8$ cells per ml. A suspension of cells in cultural medium or 0.1 M citrate-phosphate buffer was treated with NTG at 42° C (the concentration of NTG, pH of the buffer and the time of exposure are described in the text for separate experiments). Treatment with NTG was followed by two washes with an equal volume of M9 medium at 0° C and resuspension in a triple volume of medium LBM or M9M with required supplements. Part of the mutagenized culture was spread on plates containing M9MP medium and required supplements for determining the survival rate. The remaining part was grown for 2–3 h at 42° C, followed by concentration of the cell suspension by centrifugation. These cells were resuspended on M9 medium and aliquots spread as follows: on plates with M9MP medium and required supplements for determining the number of viable cells and on plates with M9MP medium without one amino acid for determining reversion frequency. For isolating auxotrophic mutants NTG mutagenesis was applied in conditions optimal for *M. flagellatum* KT (see Results).

Determination of enzyme activities of histidine and aromatic amino acid biosynthesis

Wild type strain MFK1 and mutant strains were grown for 24 h at 42° C on M9M medium with required supplements. Cells were sedimented by centrifugation (5000 rpm, 10 min), washed twice with 0.01 M Tris-HCl buffer, containing 0.2 M NaCl (pH 7.5) and resuspended with a 200-fold dilution of initial cultures in 0.01 Tris-HCl buffer with 0.006 M β-mercaptoethanol (pH 7.5). The cells were then sonicated for 1 min. The suspension of ruptured cells was centrifuged (10000 rpm, 10 min), the supernatant was used for determining the enzyme activities. All the procedures were carried out at 4° C. The activities of enzymes were determined by methods described earlier: histidinol dehydrogenase (HDG) (Ciesla et al. 1975), histidinol phosphate phosphatase (HPPT) (Ely 1974), chorismate mutase (CHM) and prephenate dehydratase (PDT) (Fiske et al. 1983). Protein concentrations were determined by the method of Lowry et al. (1951).

Results

Isolation of auxotrophic mutants on complete medium

Treatment with NTG was used for the isolation auxotrophic mutants of *Methylobacillus flagellatum* KT. After screening more than 15000 mutagenized clones grown on LBM medium, we selected several potential auxotrophic mutants. One required leucine (MFK3H), the other required valine, isoleucine and leucine simultaneously (MFK6H). The Vil⁻ phenotype was previously described for *Pseudomonas putida*

PPN (Holloway and Morgan 1986). The requirements of MFK3H and MFK6H for respective amino acids were 400–500 µg/ml. Spontaneous derivatives of these strains with lowered requirement for amino acids (100 µg/ml) were isolated (designated MFK3 and MFK6). The level of reversions to the wild type was 10^{-8} for mutation *leu*-1 and 10^{-6} for mutation *vil*-1.

Thus, as a result of this NTG-mutagenesis only two types of *M. flagellatum* KT auxotrophic mutants were obtained. Such a limited spectra of auxotrophic mutants upon selection on complete medium was observed also for other methylotrophs after mutagenesis with NTG (O'Connor et al. 1977; Gliesche and Hirsch 1986) or Tn5 (Whitta et al. 1985).

Isolation of auxotrophic mutants on supplemented minimal medium

As a result we developed a different strategy for mutant selection, which consisted for seeding the mutagenized cells on minimal medium supplemented with respective individual amino acids.

Mutagenized cultures of MFK2 were plated on M9M medium, comprising 500 µg/ml tryptophan or methionine. Of the 5000 clones that appeared on medium containing tryptophan, one required tryptophan (MFK7H) and among the 8000, that appeared on medium containing methionine, 2 required methionine (MFK8H and MFK9H). Both supplements being used at a level of 500 µg/ml.

It is worth noting that strains of *M. flagellatum* KT, particularly the auxotrophic mutants, grow very slowly on solid M9M medium, and do not always form individual colonies. The efficiency of plating was also reduced. Addition of 0.1% sodium pyruvate restores the usual efficiency of plating, the ability to form separate colonies and to a large extent stimulates the growth of *M. flagellatum* KT on minimal medium. Moreover, it reduces the differences in growth rates of the wild type strain and mutant strains. Sodium pyruvate alone can not support the growth of *M. flagellatum* KT strains but increases the growth rate of MFK1 by 19% during cultivation in liquid M9M medium (unpublished data). Growth of some type I methanotrophs has been reported to be enhanced by different multicarbon compounds (Whittenbury et al. 1970; Zhao and Hanson 1984).

Influence of complete medium on growth of auxotrophic mutants

In order to estimate the influence of complete medium on auxotrophic mutants, a comparative analysis of their growth on LBM and M9MP was carried out. Strains with a high-requirement for supplements (MFK3H and MFK6H), selected on LBM medium, grow on both media with a similar rate (time of colony formation), but their effectivity of plating on LBM medium was 5–10 times lower than on M9MP medium. Derivatives of these mutants with a lowered requirement for amino acids (MFK3 and MFK6) grow on LBM and M9MP media at equal rates and to a similar titre. The growth rate of strains with a high-requirement in nutrients (MFK7H and MFK8H), selected on M9M medium was sufficiently lower on LBM medium, and the efficiency of plating was 5–10 times lower than on M9MP medium. Many of the polyauxotrophic mutants obtained later on the basis of MFK3 were fully incapable of growth on LBM medium.

Table 2. Effect of supplements on plating efficiency of *M. flagellatum* KT strains[a]

Supplement	Concentration (µg/ml)	Plating efficiency of strains (%)		
		MFK1	MFK5	MFK3
Arg	400	170	n[b]	10
	200	n	n	50
	100	n	n	50
Trp	400	160	n	20
	200	n	n	30
	100	n	n	50
Lys	400	150	n	50
	200	n	n	50
	100	n	150	n
Met	400	n	n	50
	200	170	n	50
	100	190	170	n
Ile	400	n	n	50
	200	190	n	50
	100	190	150	n
Thr	400	n	0.1	5
	200	n	0.1	n
	100	150	n	n
Gly	400	n	0.1	0.1
	200	200	n	0.1
	100	150	n	n
Cys	400	0.1	0.1	0.1
	200	0.1	0.1	0.1
	100	10	0.2	50

[a] The cell suspensions were seeded on dishes with M9MP medium comprising one of the supplements (100, 200 or 400 µg/ml)
[b] n, supplement has no effect or influence the plating efficiency to less than 50%

Effect of nutrient supplements on growth of M. flagellatum KT strains

In order to estimate the influence of amino acids and nucleotides on *M. flagellatum* KT we used strains MFK1 (wild type), MFK3 (Leu^- derivative of MFK1 with lowered requirement for Leu) and MFK5 (spontaneous Leu^+ revertant of MFK3). The effect of nutrient supplements on the growth of these strains were evaluated by the plating efficiency and the growth rate (see the legends to Table 2 and 3). All the nutrients can be subdivided into several groups according to their effect on MFK1, MFK5 and MFK3.

Asp, Tyr, Leu do not influence significantly the growth of these strains.

Gln, Pro (100 µg/ml) increase the plating efficiency of MFK1 by 50–60%. Asn, Ala (400 µg/ml) have a similar effect on MFK5.

Arg, Ile, Lys, Met, Trp have a stimulatory effect on MFK1 and/or MFK5, but decrease the MFK3 plating efficiency (Table 2).

His, Val, Ura (400 µg/ml); Phe (200, 400 µg/ml); Glu (200 µg/ml) decrease the plating efficiency of MFK3 by 50–60% and Glu, Ade (400 µg/ml) by 80–90%.

Ser (200, 400 µg/ml) reduces the plating efficiency of MFK5 by 70–80%.

Table 3. Effect of supplements on the growth rate of *M. flagellatum* KT strains[a]

Supplement	Concentration (μg/ml)	Growth rate of strains MFK5	MFK3
Ala	400	n	— —[b]
	200	n	—
Trp	400	n	—
	200	n	—
Ile	400	n	—
Val	400	n	— —
	200	n	—
Thr	100	—	n
Ade	400	—	—
Met	400	— — —	—
	200	—	n
	100	—	n
Phe	400	— —	—
	200	— —	n
	100	— —	n
Gly	200	— — — —	— — — — —
	100	— — —	— — — —

[a] Normally the strains studied form colonies of 1—2 mm at the 2nd day of growth
[b] The growth rate was evaluated by the time of microcolony appearance: —, microcolonies at the 2nd day of growth; — —, microcolonies at the 3d day of growth etc.

Thr and Gly in low concentrations increase the MFK1 plating efficiency. These amino acids have no effect on MFK1 and completely inhibit the growth of MFK5 at high concentrations. Thr and Gly repress also the growth of MFK3: Thr has a more pronounced effect on MFK5 and Gly — on MFK3 (Table 2).

Cys in high concentrations completely inhibits the growth of MFK1 and MFK5 and strongly reduces the MFK3 plating efficiency. The reduction of the plating efficiencies of these strains is observed upon decreasing the concentration of Cys (Table 2).

Some supplements, which insignificantly decrease the growth rate of MFK1, have a far more stronger effect on MFK5 and MFK3 (Table 3). MFK5 is more susceptible to the inhibitory action of Met, Phe and Thr, while MFK3 — of Gly. Ala, Trp, Val, Ile reduce the growth rate of MFK3 only.

Thus, some nutrients, which have no or little inhibitory influence on MFK1, completely or significantly repress the growth of MFK5. At the same time most of the supplements studied inhibit the growth of MFK3, but not MFK5, or have a more pronounced effect on MFK3. Diminishing of the supplement concentrations causes reduction of the inhibitory effects (Tables 2 and 3). Moreover the inhibitory action of some supplement on MFK5 is prevented or decreased upon adding any other supplement to the growth medium. For example, the inhibition of MFK5 growth at Thr (200 μg/ml) can be completely overcome by adding Leu (100 μg/ml), Met (100 μg/ml), Trp (200 μg/ml) or Ile (400 μg/ml). Arg (400 μg/ml) and Lys (400 μg/ml) restore the plating efficiency of MFK5 by 20—40% only. The inhibitory action of the supplements on MFK3 is prevented upon increasing the concentration of Leu in the growth medium. For example, increasing the concentration of Leu from 100 μg/ml to 400 μg/ml in the medium comprising Gly (200 μg/ml) leads to a complete restoration of the plating efficiency and to a significant restoration of the growth rate of MFK3.

We studied also the influence of mixtures of nutrients, according to Holliday (1956), containing 100 or 200 μg/ml of each supplement on the growth of MFK3. Mixtures Ala, Cys, Thr, Met (200 μg/ml of each supplement) and Gly, Ser, Cys (200 μg/ml each) completely inhibit the growth of MFK3; Phe, Ser, Trp, Tyr (200 μg/ml each) and Gly, Ile, His, Lys, Val (200 μg/ml each) strongly decrease the growth rate; Ade, Phe, Ala, Arg (200 μg/ml each) — reduce the plating efficiency by 70—80%. Mixtures containing 100 μg/ml of each supplement do not inhibit significantly the growth of MFK3 and its polyauxotrophic derivatives (MFK11, MFK13, MFK18, MFK19).

Optimization of conditions for NTG mutagenesis

Isolation of several auxotrophic mutants allowed us to study the lethal and mutagenic effect of NTG on *M. flagellatum* KT quantitatively and to determine the conditions of maximal mutagenic effect. For confirming the general results obtained in experiments on optimization of NTG mutagenesis on strain MFK3, similar studies were performed also with mutants MFK12 and MFK10 (derivatives of MFK3 and MFK6, respectively), obtained in optimized conditions of mutagenesis.

With the aim of optimizing *M. flagellatum* KT NTG mutagenesis, we studied the action of factors influencing the effectivity of NTG mutagenesis of genetically well-studied microorganisms (Miller 1972). The effect of mutagen dosage was studied in a broad interval of time and concentrations. The maximal frequency of reversions for all markers was achieved during a 1 h exposure with 150 μg/ml NTG (Fig. 1, Table 4). The survival of the studied strains of *M. flagellatum* KT was significantly lower than for other bacteria, for example *Escherichia coli* (Adelberg et al. 1965).

A maximal level of mutations of *E. coli* strains upon treatment with NTG was obtained by using buffers pH 5.0—6.0 (Miller 1972). The influence of pH on the yield of reverse mutations and viability of *M. flagellatum* KT was studied in analogous conditions. The highest level of reversions was observed at pH 5.4 (Table 5). Changes in buffer pH from 5.0—7.0 caused a significant decrease in survival. Moreover, the pH of the medium directly affects the viability of cells: the survival of bacteria, when incubated for 1 h in buffer pH 5.0 was three times higher than in buffer pH 7.0. It is worth mentioning that the viability of cells, treated with NTG in culture liquid (pH 6.0—6.2) was significantly higher than after mutagenesis in citrate-phosphate buffer with the same pH. The level of revertants being practically similar.

The presence of 50 μg/ml chloramphenicol (Cm) in the medium for mutagenesis during treatment of *E. coli* K12 with low concentrations of NTG leads to an increase of the frequency of direct and reverse mutations from 5 to 50 times, with only a slight decrease in survival (Sklar 1978). High concentrations of Cm (250—500 μg/ml) increase the frequency of reversions 1.5—2 times and reduce the survival of MFK3 mutagenized cells to the same extent (Fig. 2). An analogous effect was observed upon lowering the concentration of NTG to 3 μg/ml. Cm in a concentration of 50 μg/ml, which increases the effectivity of NTG mutagenesis of *E. coli* K12, has a directly opposite effect on MFK3, when

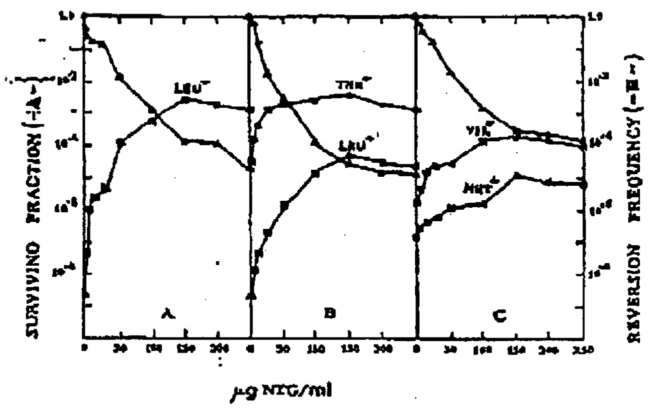

Fig. 1A–C
Survivals and reversion frequencies of strains of *Methylobacillus flagellatum* MFK3, MFK12, MFK10 as function of NTG concentration. A strain MFK3; B strain MFK12; C strain MFK10. The cell suspensions were treated with different concentrations of NTG in culture liquid for 1 h

Table 4
Survival and reversion frequency of *M. flagellatum* MFK3 as function of incubation time with NTG[a]

Duration of treatment (min)	15	30	45	60	75
Survival	1.7×10^{-2}	1.7×10^{-3}	1.6×10^{-3}	2.7×10^{-4}	1.5×10^{-4}
Frequency of leu⁺ revertants	5.7×10^{-4}	2.1×10^{-3}	2.4×10^{-3}	3.5×10^{-3}	1.1×10^{-3}

[a] The suspensions of strain MFK3 cells were treated with NTG by adding 150 μg/ml to the culture liquid for different time intervals

Table 5
Survival and reversion frequency of NTG-treated *M. flagellatum* MFK3 as function of pH[a]

pH	k[b]	5.0	5.4	6.0	6.4	7.0
Survival	2.8×10^{-4}	3.2×10^{-5}	2.0×10^{-5}	1.3×10^{-5}	3.0×10^{-6}	5.5×10^{-7}
Frequency of leu⁺ revertants	2.8×10^{-3}	2.6×10^{-3}	9.2×10^{-3}	2.4×10^{-3}	2.2×10^{-3}	2.0×10^{-3}

[a] The suspension of strain MFK3 cells in culture liquid or 0.1 M citrate-phosphate buffer with different pH values were treated for 1 h with 150 μg/ml NTG
[b] In culture medium

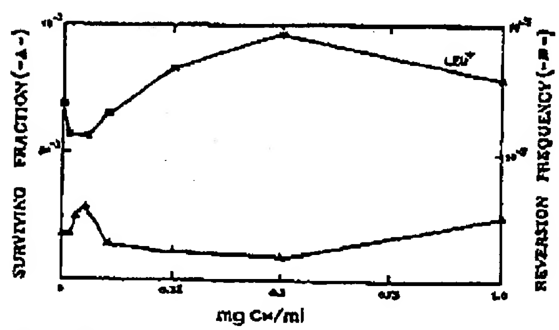

Fig. 2. Effect of Cm concentration on survival and reversion frequency of NTG-treated strain *M. flagellatum* MFK3. The suspensions of MFK3 cells in culture liquid were treated with 150 μg/ml NTG for 1 h in the presence of a range of Cm concentrations

a decrease in the frequency of reversions is accompanied by a slight increase in cell survival.

The combination of factors that most increased the level of reversions led to a total increase of mutagenesis effectivity to 8–18 times for individual markers (Table 6).

Isolation of polyauxotrophic mutants

Most of the supplements at a concentration of 100 μg/ml have no or little inhibitory effect on the growth of MFK3, that used as a parental strain for the isolation of polyauxotrophic mutants. Because of this, MFK3 cell suspensions, treated with NTG in optimal conditions, were plated on M9MP medium comprising Leu and one of the supplements in concentration of 100 μg/ml. By this approach of selection, mutants defective in the synthesis of phenylalanine (MFK18) and isoleucine (MFK19) were obtained. Met⁻-derivative of strain MFK6 (MFK10) were obtained similarly.

Mutants defective in threonine (MFK11 and MFK12) and adenine (MFK41 and MFK42) synthesis were selected only when the concentration of nutrients was augmented to 200 μg/ml. Mutants defective in histidine synthesis (MFK13, MFK15, MFK16, MFK17) were selected in analogous conditions.

It was shown further that mutants MFK13 and MFK15 have stable growth on medium comprising 100 μg/ml of histidine, while MFK16 and MFK17 require not less than 200 μg/ml histidine.

Table 6. Effects of medium, pH and Cm on survival and reversion frequency of NTG-treated *M. flagellatum* MFK3, MFK12, MFK10 strains[a]

Medium	Survival			Reversion frequency				
	MFK3	MFK12	MFK10	MFK3 leu⁺	MFK12 leu⁺	MFK12 thr⁺	MFK10 met⁺	MFK10 val⁺
b	2.0×10^{-4}	3.0×10^{-5}	4.4×10^{-4}	3.6×10^{-3}	5.4×10^{-5}	6.4×10^{-3}	2.0×10^{-4}	3.8×10^{-4}
c	2.0×10^{-5}	1.7×10^{-6}	1.8×10^{-3}	1.2×10^{-3}	1.2×10^{-4}	1.4×10^{-2}	6.0×10^{-5}	1.2×10^{-3}
d	1.0×10^{-4}	5.8×10^{-6}	6.0×10^{-5}	3.6×10^{-3}	9.6×10^{-4}	5.0×10^{-2}	2.0×10^{-4}	4.8×10^{-3}

[a] The suspensions of strains MFK3, MFK10 and MFK12 cells were treated for 1 h with 150 μg/ml NTG in culture liquid or in 0.1 M citrate-phosphate buffer pH 5.4 with 0.5 mg/ml Cm or without Cm

[b] In culture medium

[c] In 0.1 M citrate-phosphate buffer (pH 5.4)

[d] In 0.1 M citrate-phosphate buffer (pH 5.4) supplemented with 0.5 mg/ml Cm

In order to increase the effectivity of selection and for obtaining mutants defective in common stages of amino acid biosyntheses we carried out selection on M9MP medium, comprising combinations of nutrient factors with no inhibitory effect on MFK3 growth. Combination of Met, Thr (200 μg/ml each) allowed us to select only mutants defective in methionine synthesis (MFK20 and MFK21); composition of Trp, Ura, Asp (200 μg/ml) and pantothenic acid (25 μg/ml) — only mutants defective in tryptophan synthesis (MFK22 and MFK23); combination of Ade, Arg (200 μg/ml each), Bio (25 μg/ml) — only mutants defective in biotin synthesis (MFK24—MFK40). The absence of Thr⁻ mutants upon selection on media with Met, Thr is now clear, since we gained evidence that methionine in concentration 200 μg/ml strongly inhibits the growth of mutants requiring threonine. However, arginine (200 μg/ml) has no effect and biotin (25 μg/ml) even stimulates the growth of mutants requiring adenine. Thus the absence of mutants defective in adenine synthesis in this particular case, is not linked directly with the inhibitory effect of supplements or the insufficient concentration of adenine. Mutants MFK20, MFK21, MFK22 and MFK23 have a stable growth on medium, containing 100 μg/ml required nutrients. The stable growth of mutants MFK24—MFK40 was provided by 10 μg/ml biotin.

Using strain MFK14 grown in the presence of 100 μg/ml of methionine, we obtained mutant MFK43 that was used in the following experiments for isolation of mutants with four auxotrophic mutations, using combinations with nutrient supplements (100 μg/ml each). Combination of Ade, Phe, Ala, Arg allowed the selection of mutants defective in alanine (MFK44) and phenylalanine (MFK47) syntheses; combination of Gly, Ile, Val, Ura — mutants, requiring isoleucine (MFK45) and isoleucine and valine simultaneously (MFK46); combination of Gly, Ser, Cys — mutants defective in glycine and serine with high frequencies of reversions (10^{-3}—10^{-4}); combination of Phe, Tyr, Trp, Ser — mutants, requiring phenylalanine (MFK48), tyrosine (MFK49) or both, simultaneously (MFK50). All the isolated mutants required 100 μg/ml of the corresponding supplements, while MFK50 required 200 μg/ml tyrosine.

Mutant MFK50, that requires phenylalanine and tyrosine simultaneously, most probably carries two mutations in phenylalanine and tyrosine syntheses. This is indicated by different levels of reversions on media, that comprise only phenylalanine or tyrosine, and the absence of revertants on media depleted of both amino acids. This supposition is confirmed also by the data on the activity of enzymes involved in the syntheses of aromatic amino acids.

Upon checking the mutants MFK47 and MFK48 requiring phenylalanine, we revealed that mutations *met-4* and *hisB1* of parent strain MFK43 in these mutants reverted to the wild type. Similarly, in mutants MFK41 and MFK42, requiring adenine, mutation *leu-1* reverted to the wild type.

The frequency of isolation of auxotrophic mutants varies from 0.54% for Bio⁻ mutants to 0.02% for Ilv⁻ mutants. The levels of spontaneous reversions to the wild type are 10^{-6}—10^{-9} for different auxotrophic markers.

Determination of metabolic defects in some auxotrophic mutants

By studying the growth of mutants requiring tryptophan on media comprising indole or anthranilate, we revealed that strains MFK7H and MFK23 grow only on indole, while strain MFK22 grows on indole or anthranilate. A concentration of 10 μg/ml of precursors is sufficient for normal growth. The ability to grow on indole or anthranilate indicates a mutation in gene *trpE*, that encodes anthranilate synthetase. Strains MFK7H and MFK23 can probably carry a mutation in genes *trpD*, *trpC* or *trpB* (Crawford 1975; Camakaris and Pittard 1971).

The activity of CHM and PDT were determined in crude extracts of strain MFK1 cells and mutants, requiring phenylalanine or phenylalanine and tyrosine simultaneously. The levels of the activity of these enzymes in *M. flagellatum* were much lower than in *E. coli* (Im and Pittard 1971). The activity of CHM was practically the same in strain MFK1 and all the mutants studied — 0.5—0.6 nmol/min · mg protein. The activity of PDT was lower in mutants compared to 0.8—0.9 nmol/min · mg protein in the wild type strain: for mutant strain MFK18 it was 7%; for MFK47 — 17%; for MFK48 — 23%; for MFK50 — 27%, all indicating a mutation in gene *pheA*.

The activities of HDG and HPPT, two enzymes of histidine biosynthesis were determined in crude extracts of strain MFK3 and their derivatives, requiring histidine (MFK13, MFK15, MFK16, MFK17). The levels of enzyme activities of *M. flagellatum* and *E. coli* (Garrick-Silversmith and Hartman 1970) were similar. The activity of HDG in strain MFK3 was 1.06 nmol/min · mg protein, the activity of HPPT — 1.26 nmol/min · mg protein. All the mutants

reveal the activity of HDG (1.06–4.06 nmol/min · mg protein). The levels of HPPT activity were 0.74, 3.20, 1.36 nmol/min · mg protein in strains MFK15, MFK16, MFK17, respectively. The activity of HPPT was completely absent in mutant MFK13, which suggest a mutation in gene *hisB* (Goldschmidt et al. 1970).

Discussion

The reasons preventing the isolation of auxotrophic mutants of methylotrophic bacteria can be strain-specific and common for all or several methylotrophs. From our point of view the selection of auxotrophic mutants on complete media seems to be one of the common reasons at least for those obligate and facultative methylotrophs for wich a limited range of auxotrophs upon selection on complete medium was observed (O'Connor et al. 1977; Tatra and Goodwin 1983; Gliesche and Hirsch 1986). Inhibition of the growth of mutants by components of complete medium was suggested by Whitta et al. (1985) for explaining the limited spectrum of auxotrophic mutations upon Tn*5* induced mutagenesis in facultative methylotroph *Methylobacterium AM1*.

Studies on the obligate methylotroph *M. flagellatum* KT carried out in the present study indicated that some of the nutrient supplements and some their mixtures to have inhibitory effect on growth of *M. flagellatum* KT strains. We demonstrated that prototroph (MFK*5*) obtained from auxotrophic mutant with lowered requirement for Leu (MFK3) is more susceptible to the inhibitory action of the same nutrients, than wild type strain (MFK1). We suppose that MFK1 has a low permeability for the growth factors, therefore the availability of the essential nutrients in LBM medium can be insufficient for some auxotrophic mutants originated from wild type strain. The mutation of lowered requirement for Leu most probably causes increasing of the permeability not only for Leu, but for all or most of the nutrients. We demonstrated also that most of the nutrients have far more stronger inhibitory effect on auxotroph MFK3, than on isogenic prototroph MFK*5*. Hence the inhibition by nutrients can lead to a death of some potential auxotrophs and limitation of the spectrum of auxotrophic mutations.

Thus, the influence of complete medium on the selection of auxotrophic mutants *M. flagellatum* KT can be explained by two processes-inhibition and starvation for nutrients. Inhibition can be linked with the effect of nutrient supplements on metabolic regulation. Such a possibility was shown for methanotrophs *Methylococcus capsulatus* (Eccleston and Kelly 1973) and *Methylococcus thermophilus* (Pinchuk 1986). Indeed, some of the amino acids completely or partially inhibit the growth of wild type strain and prototroph MFK*5*. Starvation for growth factors can be provoked by two reasons — an insufficient concentration of some nutrients in the complete medium and/or by competition between the growth factors for penetrating the cell. Such a competition is characteristic for microorganisms that transport their nutrients by facilitated diffusion, via the low-affinity system of active transport, or by a combination of both processes (Eccleston and Kelly 1972). Some results indicate that this is true for *M. flagellatum* KT. Firstly, the inhibition of the growth of prototroph MFK*5* can be prevented by adding any other supplement to the growth medium, and secondly, the inhibition of the growth of auxotrophic mutant MFK3 in the presence of different nutrient supplements is completely recovered by increasing the concentration of Leu.

Thus, the isolation of auxotrophic mutants from arbitrary methylotrophic bacteria can be based on the consecutive selection of mutants for individual genes using mineral media supplemented with high concentration of corresponding nutrient. These experiments must involve the analysis of the inhibitory effect of nutrient supplements on the growth of the wild type strain and in particular, of the mutant strains. This approach has allowed us to isolate auxotrophic mutants of the restricted facultative methylotroph Organism W3A1 (Colby and Zatman 1975) and methanotroph *Methylomonas methanica* (Trotsenko 1976).

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